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Monitoring of Epstein-Barr virus load after hematopoietic stem cell transplantation for early intervention in post-transplant lymphoproliferative disease

Astrid Meerbach[1*], Peter Wutzler[1], Ralf Häfer[2], Felix Zintl[2], Bernd Gruhn[2]

[1]Institute of Virology and Antiviral Therapy, Medical Center, Friedrich-Schiller University Jena, Jena, Germany

[2]Department of Pediatrics, Medical Center, Friedrich-Schiller University Jena, Jena, Germany

*Corresponding author:

Dr. med. Astrid Meerbach, Institute of Virology and Antiviral Therapy, Medical Center, Friedrich-Schiller University Jena, Hans-Knöll-Strasse 2, D-07745 Jena, Germany

phone.: +49-3641-657300; fax: +49-3641-657301

E-mail address: Astrid.Meerbach@med.uni-jena.de

Running head: Monitoring Epstein-Barr virus load

ABSTRACT

Epstein-Barr virus (EBV)-associated post-transplant lymphoproliferative disease is a life-threatening complication following hematopoietic stem cell transplantation. A quantitative polymerase chain reaction to evaluate EBV-genome copy numbers based on a nested polymerase chain reaction and an end-point dilution was used. Applying this assay EBV load was prospectively screened weekly in 123 patients after transplantation. The results demonstrate that EBV reactivations with more than 1,000 EBV-genome copies measured in 10^5 peripheral blood mononuclear cells were observed in 31 patients (25.2%). Three patients developed lymphoproliferative disease with extremely high EBV-genome copies in peripheral blood mononuclear cells (>100,000 copies/10^5 cells) and plasma. After combined antiviral and immune therapy two of three patients showed a dramatic decrease of EBV load and survived, while the third patient died of lymphoma. A subclinical EBV reactivation was observed in 24 cases (19.5%) with EBV-genome copies in 10^5 peripheral blood mononuclear cells ranging between 2,500 and mostly 10,000. After reduction of immunosuppression the EBV levels normalized. In four patients, the high copy number of ≥80,000 copies/10^5 peripheral blood mononuclear cells and plasma positivity prompted us to start pre-emptive therapy with rituximab and cidofovir for prevention of lymphoproliferative disease. After drug administration the high EBV load was reduced remarkably. Ninety-two patients (74.8%) who had ≤1,000 copies/10^5 peripheral blood mononuclear cells did not develop EBV-associated lymphoproliferative disease. In conclusion, monitoring of EBV load is a sensitive and useful parameter in the surveillance of EBV reactivation for early intervention in EBV-associated lymphoproliferative disease as well as for follow-up of the efficacy of therapy.

Keywords: Epstein-Barr virus (EBV), monitoring EBV load, post-transplant lymphoproliferative disease, prevention, therapy

Introduction

The Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and has been found in association with several human malignancies [for review, see Andersson, 2000; Young and Rickinson, 2004]. Tumors classically linked with EBV are the Burkitt´s lymphoma [Burkitt, 1958; Epstein et al., 1964] and the nasopharyngeal carcinoma [zur Hausen et al., 1970]. However, EBV was also found to be involved in the development of other neoplasias, such as B-cell lymphomas in immunocompromised transplant [Hopwood and Crawford, 2000] and AIDS patients [Hamilton-Dutoit et al., 1991; Shibata et al., 1993], certain T-cell lymphomas [Jones et al., 1988; Su et al., 1991; Meijer et al., 1996], Hodgkin´s lymphomas [Staal et al., 1989; Herbst et al., 1990; Niedobitek, 1996] and gastric carcinomas [Leoncini et al., 1993; Fukayama et al., 1994; Osato and Imai, 1996].

Patients receiving immunosuppressive therapy after organ and hematopoietic stem cell transplantation run a particularly high risk of developing EBV-associated post-transplant lymphoproliferative disease, which may take a rapid fatal course. Because of the growing number of high-risk transplantations the incidence of post-transplant lymphoproliferative disease is increasing. Several authors summarized the current knowledge of EBV-induced post-transplant lymphoproliferative disease with regard to the varying incidence, risk factors, heterogeneous clinical presentation, histological findings and treatment modalities [reviews: Purtilo et al., 1992; Savage and Waxman, 1997; Lucas et al., 1997; Paya et al., 1999; Wagner et al., 2002a; Cohen, 2003; Loren et al., 2003; Meerbach et al., 2004]. Post-transplant lymphoproliferative disease can differ clinically from a mononucleosis-like syndrome to malignant lymphoma. The incidence varies between 0.5 and 20% depending on several risk

factors, particularly donor-recipient human leukocyte antigen mismatch, T-cell depletion of donor graft or use of antithymocyte globulin. Despite different treatment modalities including reduction of immunosuppression, conventional chemotherapy, administration of antiviral drugs such as aciclovir, ganciclovir, foscarnet, and cidofovir or hyperimmune globulin and interferon-α, adoptive immunotherapy with donor lymphocytes or EBV-specific cytotoxic T lymphocytes as well as the use of anti-B cell monoclonal antibodies the mortality rate is still high. Therefore, prevention of EBV-associated post-transplant lymphoproliferative disease will be the main goal. To make this approach practical and successful, two points are important. First, it is necessary to identify “high-risk” patients. And secondly, sensitive and reliable methods have to be available for detecting EBV reactivation and early onset of post-transplant lymphoproliferative disease.

Serologic data, frequently used to diagnose viral infections, represent only indirect markers of infection and are often unreliable in immunocompromised patients. Qualitative polymerase chain reaction assays are unable to distinguish between active and latent infection. As reviewed by Rowe et al. [2001] quantitative polymerase chain reaction of EBV DNA in peripheral blood is a highly sensitive and specific method for monitoring transplanted patients.

The present study was aimed at monitoring EBV load in plasma and peripheral blood mononuclear cells in 123 patients undergoing hematopoietic stem cell transplantation.

PATIENTS, MATERIAL AND METHODS

Patients

In the prospective follow-up study of EBV load, 123 patients were included who underwent hematopoietic stem cell transplantation in the Department of Pediatrics at the University Jena between January 2000 and August 2006. Children who died within the first 8 weeks after stem cell transplantation because of underlying disease or other non-EBV-associated complications were not included in the study. Hematopoietic stem cell transplantation was performed because of hematological diseases in 70 patients, oncological diseases in 38 patients, rheumatological diseases in 8 patients and genetic diseases in 7 children. Eighty-three patients received an allogeneic stem cell transplantation from an unrelated donor (51 patients) and from a related donor (32 patients), respectively. Forty patients were treated by autologous stem cell transplantation. Thirteen patients underwent two or more transplantations. The patient group consisted of 48 female and 75 male patients with a median age of 12 years (range: 0.2-25 years). The EBV serostatus of all patients was evaluated before transplantation. EBV load in plasma and peripheral blood mononuclear cells was prospectively monitored once weekly. If there were no signs of EBV reactivation until day +100, monitoring was continued monthly up to one year after stem cell transplantation.

Clinical Samples and Controls

Patient samples

A total of 1,393 plasma samples, 2,827 samples of peripheral blood mononuclear cells, 14 samples of cerebrospinal fluid and 18 tissue samples were investigated by polymerase chain reaction. EDTA-blood (5 ml) was used for plasma extraction and

isolation of peripheral blood mononuclear cells. Blood samples were purified by Ficoll-Hypaque density gradients using Leucosep tubes (Greiner) for 15 min at 800*g*. Plasma was removed and repeated cleared by centrifugation for 20 min at 300*g*. The layer of mononuclear cells was carefully aspirated, washed three times with phosphate-buffered saline and sedimented by centrifugation at 250*g* for 10 min. The cell pellet was resuspended in 200 µl phosphate-buffered saline and cells were counted using a Neubauer chamber. Occasionally, 200 µl of native cerebrospinal fluid or up to 25 mg tissue were used for DNA extraction.

Controls

The EBV-genome negative cell line Ramos as well as an EBV p23 plasmid served as controls. The plasmid standard was generated as described recently by Krumbholz et al. [2006].

DNA Extraction

DNA was extracted from plasma, peripheral blood mononuclear cells, cerebrospinal fluid and the EBV-negative control cell line Ramos by QIAamp DNA Blood Mini Kit (Qiagen). For tissue DNA isolation the QIAamp DNA Mini Kit (Qiagen) was used. DNA was extracted according to the manufacturer's instructions and DNA concentration was determined by UV spectrometry.

Quantitative EBV DNA analysis

The method was described in detail previously [Meerbach et al., 2001]. The following steps were performed:

Preparation of dilution rows for samples and control standard

For detection of EBV-genome copies by polymerase chain reaction 5 µl of DNA from plasma or cerebrospinal fluid as well as DNA from peripheral blood mononuclear cells was used in concentrations of 1 µg, 500 ng and 100 ng. In case of positive polymerase chain reaction signal a dilution row was prepared by the factor 2 to at least 1:128 (plasma, cerebrospinal fluid) and 1 ng (peripheral blood mononuclear cells), respectively. From EBV plasmid DNA serial 10-fold dilutions were prepared from 10^8 to 10^{-1} copies/5 µl.

Polymerase chain reaction

The polymerase chain reaction for detecting the EBV sequences was performed using primers of the virus capsid antigen p23 region. The primer pair p23-1, p23-2 was used to amplify a 482 base pairs fragment of the BLRF2 gene of EBV [Reischl et al., 1996]. The sequences are as follows: p23-1: 5´ATCAGAAATTTGCACTTT CTTTGC3´, p23-2: 5´CAGCTCCACGCAAAGTCAGATTG3´. For the detection of EBV-specific polymerase chain reaction products a set of internal primers (p23-3, p23-4) was used for nested PCR which amplifies a 363 base pairs segment. They have the following sequences: p23-3: 5´TTGACATGAGCATGGAAGAC3´, p23-4: 5´CTCGTGGTCGTGTTCCCTCAC3´. A total volume of 50 µl reaction mixture consisted of polymerase chain reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl at pH 8.3), 200 µM dNTP, 1 µM of each primer, 0.6 units Taq DNA polymerase (Roche), and 5 µl of DNA solution. After an initial denaturation step for 5 min at 94°C, 30 cycles were performed each including 45 sec at 94°C for denaturation, 45 sec at 60°C for annealing, and 1.5 min at 72°C for extension, followed by an additional 7 min extension at 72°C. For the nested polymerase chain reaction, 1 µl of the polymerase chain reaction product from the first series of amplifications was

added to 49 µl of the polymerase chain reaction reaction mixture and amplified in additional 30 cycles.

Agarose gel electrophoresis of viral DNA

A 10 µl sample of the amplification product was run on a 0.8% agarose gel electrophoresis. DNA was monitored under UV light after staining with ethidium bromide. The last visible band in each dilution row corresponds to about 10 viral copies (Figure 1).

Interpretation

To calculate the number of EBV copies in the initial DNA sample (1 µg of cell DNA or 20 µl of plasma or cerebrospinal fluid, respectively) the dilution factor was multiplied by 10 (sensitivity level of polymerase chain reaction: approximately 10 EBV-genome copies). For plasma and cerebrospinal fluid, the result was multiplied by 50 to obtain copies per ml. For peripheral blood mononuclear cells, the calculation is based on the presumption that 1 µg total DNA represents approximately 10^5 cells [Knecht et al., 1992]. Polymerase chain reaction-positive plasma/cerebrospinal fluid samples and/or EBV-genome copy numbers >1,000 in 10^5 peripheral blood mononuclear cells were estimated as active EBV infection.

Flow cytometry

Percentage of lymphocytes was calculated by flow scan analysis with the Coulter Epics XL-MCL instrument (Coulter/Beckman). Monoclonal antibodies produced by Dako were used according to the manufacturer's instructions. B lymphocytes were detected by anti-$CD19^+/CD22^+$ and anti-$CD19^+/CD22^-$ antibodies. An anti-$CD3^+$ antibody served for the detection of T cells. Suppressor/cytotoxic T cells and helper T

cells were counted by application of anti-$CD8^+/CD3^+$ and anti-$CD4^+/CD3^+$ antibodies, respectively.

Antibody detection

Patients sera were tested for antibodies to structural antigens (virus capsid antigen) and for antibodies to the early antigen complex of EBV by immunofluorescence according to the method described by Henle and Henle [1966]. In addition, antibodies to the recombinant protein Epstein-Barr nuclear antigen 1 rp72 were detected by ELISA (Biotest).

RESULTS

EBV DNA load monitoring after hematopoietic stem cell transplantation

EBV reactivations and primary infections, respectively with >1,000 EBV genome copies in 10^5 peripheral blood mononuclear cells were observed in 31 out of 123 patients (25.2%) between days 19 and 303 (median day: 51) after stem cell transplantation. The increase of EBV-genome copies was found at least two weeks before reactivation from undetectable concentrations in peripheral blood mononuclear cells via low copy numbers (10-100 copies/10^5 peripheral blood mononuclear cells) to intermediate values (≤1,000 copies/10^5 peripheral blood mononuclear cells) up to reactivation with >1,000 EBV-genome copies/10^5 peripheral blood mononuclear cells. Only one of the 31 patients (patient 64) with >1,000 EBV genome copies in 10^5 peripheral blood mononuclear cells was EBV-seronegative before transplantation suggesting a primary infection. From 31 patients developing an active EBV infection 26 (83.9%) had been treated with antithymocyte globulin. In 8 patients demonstrating elevated EBV levels T cell depletion in the transplant was performed by positive selection of $CD34^+$ progenitor cells. Characteristics of the patients with EBV reactivation are summarized in Table 1. Statistical calculations of cut-off values for EBV lymphoproliferative disease as well as reactivation are shown in Table 2 and 3, respectively.

Three patients (2.4%) developed an EBV-associated post-transplant lymphoproliferative disease with extremely high EBV copy numbers in plasma and peripheral blood mononuclear cells. In these patients, anti-EBV antibodies could be detected before transplantation. Thus, development of lymphoma was due to EBV reactivation. A strong increase of EBV-genome copies was observed 1-4 weeks before the onset of clinical signs. The high EBV load in plasma and peripheral blood mononuclear cells reduced dramatically after therapy in two cases, while the third

patient died of lymphoma. The decrease of EBV-genome copies was associated with a decrease of B lymphocytes and an increase of $CD3^+$, $CD8^+/CD3^+$ and $CD4^+/CD3^+$ T lymphocytes. In the following the three cases of EBV-associated post-transplant lymphoproliferative disease are reported in detail:

Patient 3

A 11-year-old girl with chronic myeloid leukemia underwent allogeneic stem cell transplantation from an unrelated donor. For prophylaxis of graft-versus-host-disease, she was given antithymocyte globulin, cyclosporine A and methotrexate. Ninety days after transplantation, she presented with fever, exsudative tonsillitis and lymph node swelling. A lymph node biopsy revealed an immunoblastic lymphoma positive for the proliferation marker Ki67 as well as for the CD20 antigen. By in situ hybridization EBV-encoded RNA (EBER) could be detected in the tumor cells. On day 81 after stem cell transplantation, she was found to have an extremely high EBV load (1.0×10^6 EBV-genome copies/10^5 peripheral blood mononuclear cells). The EBV-genome copies still increased to $13 \times 10^6/10^5$ peripheral blood mononuclear cells and 510,000/ml plasma on day +100. Also in the lymph node high EBV values were measured (13×10^6 copies/µg DNA). Despite of stop of immunosuppressive drugs and therapy with ganciclovir and foscavir, administration of anti-CD20 monoclonal antibody rituximab and donor lymphocyte infusions, the general condition deteriorated remarkably. Pulmonary insufficiency required artificial respiration. After progressive insufficiency of circulation, renal and liver failure she died of multiorgan failure on day 111 after stem cell transplantation.

Patient 8

A 12-year-old boy with acute lymphoblastic leukemia received an allogeneic stem cell transplantation from an unrelated donor. To prevent graft-versus-host-disease, he was given antithymocyte globulin, cyclosporine A and methotrexate. On day 66 after stem cell transplantation, he developed fever, inguinal and cervical lymphadenopathy and angina lacunaris. Histological examination revealed an immunoblastic B cell lymphoma. Tumor cells were positive for Ki67 and CD20 antigens as well as EBV-encoded RNA (EBER). The active EBV infection could be confirmed by polymerase chain reaction detecting 4,000 EBV-genome copies/ml plasma, 500 copies/ml cerebrospinal fluid and 16,000 copies/10^5 peripheral blood mononuclear cells. A B cell peak was seen on day +71 with 9.5% corresponding to 32 lymphocytes/µl. The antiviral and immune therapy consisted of withdrawal of cyclosporine A, continuation of ganciclovir and addition of cidofovir, rituximab and donor lymphocyte infusions. On day +73 the patient developed an acute pulmonary insufficiency managed by high-dose O_2 administration and methylprednisolone. The increasing EBV load (Figure 2A) reached highest values on day +82 (16,000 copies/ml plasma; 300,000 copies/10^5 peripheral blood mononuclear cells) and +84 (Figure 1). The boy presented still with fever, reduced general condition, diffuse changing pain, sickness, vomiting and fatigue. On day +88, for the first time T cells could be detected by flow cytometry increasing up to 17% $CD3^+$ lymphocytes (Figure 2B) corresponding to 349 cells/µl on day +92. $CD8^+/CD3^+$ T cell number increased to 14.5% (Figure 2B) corresponding to 411 cells/µl on day +95. A clinical worsening occurred with somnolence, confusion and central apnea. EBV polymerase chain reaction revealed 1,000 copies/ml cerebrospinal fluid. The patient was additionally treated with dexamethasone and aminophylline. On day 114 after stem cell transplantation the boy developed an acute graft-versus-host-disease of skin, liver and intestine, which could

be successfully treated with methylprednisolone, cycosporine A, mycophenolate mofetil and antithymocyte globulin. Clinical signs of graft-versus-host-disease occurred in line with rising numbers of $CD3^+$ and $CD8^+/CD3^+$ T lymphocytes. The therapy of the post-transplant lymphoproliferative disease resulted in improvement of his general condition, resolution of fever, involution of lymphadenopathy and angina lacunaris. EBV-genome copies in plasma and peripheral blood mononuclear cells decreased remarkably to undetectable levels on day +119 (Figure 2A).

Patient 21

A 16-year-old boy with systemic lupus erythematodes received an autologous stem cell transplantation. On day +41 his general condition deteriorated with fever, sore throat and difficulty in swallowing. An ulcerative tonsillitis, cervical lymph node swelling and septical temperatures occurred 50 days after transplantation. The histological examination of lymph node biopsy revealed a non-Hodgkin lymphoma of high-grade malignancy with EBV-encoded RNA (EBER) detection as well as expression of the ZEBRA protein. First antiviral therapy consisted of aciclovir and ganciclovir. Because of further increasing EBV load (Figure 3A) with the highest values on day +55 (2,000 copies/ml plasma; 1.7 x 10^6 copies/10^5 peripheral blood mononuclear cells) the antiviral therapy was changed to cidofovir in combination with rituximab. In the following days the EBV load reduced dramatically (Figure 3A). In Figure 3B the elevated B cell counts are demonstrated showing a peak on day +54 with 73.1% corresponding to 2,778 B cells/µl. The decline of EBV-genome copies and B cells was accompanied with a reconstitution of T cell immunity. T cells increased from 3% on day +55 to 27% $CD3^+$ T cells corresponding to 1,291 cells/µl and 28% $CD8^+/CD3^+$ lymphocytes corresponding to 1,330 cells/µl on day +59, respectively (Figure 3B). From day +59 the patient became afebrile and gradually

improved. With complete recovery of clinical signs EBV-genome copies reached normal values (<10 copies/10^5 peripheral blood mononuclear cells).

In four patients, the rapidly increasing EBV load to at least 80,000 EBV-genome copies/10^5 peripheral blood mononuclear cells together with EBV plasma positivity was the reason to start pre-emptive therapy with a single dose of rituximab (375 mg/m^2) and cidofovir (5 mg/kg/week for 2 weeks) for prevention of an EBV-associated post-transplant lymphoproliferative disease. According to the increasing EBV load there was also an increase of B lymphocytes, reaching the highest value of 17.4% on day +49 in patient 22 corresponding to 230 B cells/µl (Figure 4). After administration of rituximab the high EBV load of patients 20, 22, 23 and 95 in plasma (maximum: 250,000; 1,000; 2,000; 1,000 copies/ml, respectively) and peripheral blood mononuclear cells (maximum: 80,000; 600,000; 80,000; 80,000 copies/10^5 peripheral blood mononuclear cells, respectively) decreased remarkably accompanied by a reduction of B lymphocytes.

A subclinical EBV reactivation was observed in 24 patients (19.5%) with EBV-genome copies mostly between 2,500 and 10,000/10^5 peripheral blood mononuclear cells. Only 5 of the 24 patients had a single higher peak of 20,000 or 40,000 copies/10^5 peripheral blood mononuclear cells. After reduction or stop of immunosuppression the EBV levels normalized.

The 92 patients (74.8%) who had copy numbers of ≤1,000 copies/10^5 peripheral blood mononuclear cells did not develop EBV-associated post-transplant lymphoproliferative disease.

EBV serostatus

From the 123 patients undergoing stem cell transplantation 13 children were EBV seronegative before transplantation (10.6%). For protection they were given immunoglobulins after transplantation resulting in high IgG antibody titers against virus capsid antigen and nuclear antigen of EBV.

DISCUSSION

One increasing area of concern is EBV infection and its relevance to post-transplant lymphoproliferative diseases. Post-transplant lymphoproliferative diseases encompass a spectrum of lymphoid proliferations to lymphomas that occur in recipients of solid organ and bone marrow or peripheral blood stem cell transplants in connection with immunosuppression. The development of a life-threatening EBV-associated post-transplant lymphoproliferative disease is a serious complication after transplantation. The availability of potentially effective therapeutic regimens for EBV post-transplant lymphoproliferative disease increased the necessity for early identification of the subset of patients at high risk of developing this complication. Therapeutic efforts will probably be most effective if they are introduced early in the course of this rapidly progressing disease and before the onset of overt clinical symptoms.

The aim of the performed prospective study in 123 patients undergoing hematopoietic stem cell transplantation was the monitoring of EBV load for early detection of EBV reactivation. The results indicate that EBV reactivations are frequent events after stem cell transplantation as described by van Esser et al. [2001a]. About 25% of our patients showed EBV reactivation at a median time of 51 days after stem cell transplantation. According to Dominietto et al. [2004] EBV reactivation is high (above 50%) in patients undergoing an alternative donor transplant and occurs at a median interval of 45 days from stem cell transplantation. Whereas modulation of immunosuppression was sufficient in 24 out of our 31 patients to normalize EBV levels, four patients received pre-emptive therapy with rituximab and cidofovir to prevent post-transplant lymphoproliferative disease. However, three patients (2.4%) developed post-transplant lymphoproliferative disease, of whom one died of lymphoma. By com-

bined antiviral and immune therapy two patients with post-transplant lympho-proliferative disease could be successfully treated.

Concerning the diagnosis of EBV reactivation many studies demonstrated that the quantitative EBV DNA detection correlates with EBV-caused immunopathologic changes in the development of EBV post-transplant lymphoproliferative disease [Kenagy et al., 1995; Martinez et al., 1995; Bai et al., 1997; Rowe et al., 1997; Brengel-Pesce et al., 2002; Gärtner et al., 2002; Sirvent-von Bueltzingsloewen et al., 2002]. High amounts of EBV in peripheral blood mononuclear cells were detected in these patients [Savoie et al., 1994; Rooney et al., 1995; Lucas et al., 1998]. Several authors have reported a significantly increased EBV load at the time of diagnosis of post-transplant lymphoproliferative disease, up to $4\log_{10}$ higher than in control groups [Bai et al., 1997; Riddler et al., 1994; Rowe et al., 1997; Meerbach et al., 2001]. A rapid increase in peripheral blood EBV DNA load diagnosed and predicted post-transplant lymphoproliferative disease [Stevens et al., 2001a; Van Esser et al., 2001a; Orentas et al., 2003].

In addition, quantitative polymerase chain reaction assay for EBV DNA in peripheral blood is useful in monitoring the efficacy of therapy [Hoshino et al., 2000; Meerbach et al., 2001; Gärtner et al., 2002]. Kenagy et al. [1995] diagnosed markedly elevated levels of EBV DNA in post-transplant lymphoproliferative disease during illness as well EBV DNA levels were reduced dramatically due to effective therapy. This observation is in line with our data. The EBV load of two patients with post-transplant lymphoproliferative disease increased within 4-5 weeks to maximal values of up to 1.7 million copies in 10^5 peripheral blood mononuclear cells. After combined antiviral and immune therapy the EBV load decreased rapidly to undetectable concentrations. However, despite of different therapeutic measures patient 3 died of lymphoma. She

presented distinct higher copy numbers and showed already with the first clinical symptoms 1.0 million EBV-genome copies. The copy numbers rose to 13 million and did not decrease during therapy. The high viral load appears to be a poor prognostic sign.

Curtis et al. [1999] analyzed in a long-term multi-center study incidence, risk factors and outcome of post-transplant lymphoproliferative disease. The risk for early-onset post-transplant lymphoproliferative disease, e.g. during the first year after transplantation, was strongly associated with (i) unrelated or human leukocyte antigen mismatched related donor, (ii) T-cell depletion of donor marrow, (iii) use of antithymocyte globulin or anti-lymphocyte globulin, and (iv) anti-CD3 monoclonal antibody (OKT3) for prophylaxis or treatment of acute graft-versus-host-disease. Additionally to these four main risk factors a weaker association could be demonstrated with the occurrence of graft-versus-host-disease grades II to IV and with conditioning regimens that included irradiation. Of epidemiological importance is the synergistic occurrence of several risk factors. So, the rate of post-transplant lymphoproliferative disease among patients with no risk factor was 0.5% increasing to 1.7% in the case of one risk factor. The rate reached 8% in the presence of two risk factors and increased to the highest rate of diseases of more than 20% with at least three major risk factors [Curtis et al., 1999]. An increased risk of post-transplant lymphoproliferative disease with more than one risk factor showed also the three patients with post-transplant lymphoproliferative disease in the presented study. All of them were treated with antithymocyte globulin as a main risk factor. Additionally, two of these patients were irradiated as part of the conditioning regimen. One of the EBV-seropositive patients developed a post-transplant lymphoproliferative disease after a T cell depleted $CD34^+$ selected autologous stem cell transplantation for systemic lupus erythematodes. Although the risk of EBV post-transplant lymphoproliferative

disease after autologous transplantation is low, case reports of this complication in the autologous setting exist [Chao et al., 1993, Briz et al., 1997, Hauke et al., 1998]. Recently, Powell and coworkers [2004] observed an unexpected high incidence of EBV lymphoproliferative diseases after $CD34^+$ selected autologous stem cell transplant in neuroblastoma. The authors concluded that the combination of tandem stem cell transplantation and $CD34^+$ selection may have increased immunosuppression in these patients to a point where there is an elevated risk of EBV lymphoproliferative disease. Nash et al. [2003] reported EBV-associated lymphoproliferative diseases in patients after autologous stem cell transplantation for the control of severe autoimmune diseases. The addition of antithymocyte globulin in the high-dose immunosuppressive therapy regimen in these patients as well as in our case report and $CD34^+$ selection of the autologous graft may induce a higher degree of immunosuppression with an increased risk of transplant-related complications including EBV lymphoproliferative disease.

Concerning the time point, the incidence of post-transplant lymphoproliferative disease was highest 1 to 5 months after transplantation with a peak in the third month [Curtis et al., 1999]. In several studies the median time from stem cell transplantation to diagnosis lymphoproliferative disease was 76 days [Simon et al., 1991], 71.5 days [Micallef et al., 1998] and 86 days [Gross et al., 1999], respectively. According to these data, in the presented study first clinical signs of post-transplant lymphoproliferative disease occurred on days +50, +66, and +90, respectively. Early-onset forms often show a rapid disease progression with a median survival of 0.6 months [Gross et al., 1999]. Micallef et al. [1998] described in 5 out of 8 patients a fulminant course with multiorgan failure and death within 3-8 days after onset of clinical signs. An incidence of 1.2% of fatal lymphoproliferative disease after allogeneic bone marrow transplantation was observed by Simon et al. [1991] with a

course rapidly progressive leading to death in 2-5 weeks. Such a fulminant course of post-transplant lymphoproliferative disease was also observed in patient 3, who died on day 21 after onset of clinical symptoms.

A disadvantage at present is that EBV polymerase chain reaction is not yet standardized. This is why published data differ in method, sensitivity, material and even in the copy numbers of the results discussed in the following paragraphs:

Method: Looking at the method EBV load can be detected by semiquantitative [Lukas et al., 1998], quantitative competitive [Rowe et al., 1997; Baldanti et al., 2000; Yang et al., 2000; Gärtner et al., 2002] or real-time polymerase chain reaction [Hoshino et., 2000; Wagner et al., 2002b; Orentas et al., 2003; Yancoski et al., 2004]. In the presented prospective study, the author's used a semiquantitative nested EBV polymerase chain reaction [Meerbach et al., 2001], which clearly detects 10 viral copies in 10^5 peripheral blood mononuclear cells. As reported recently [Krumbholz et al., 2006], a comparison of the semiquantitative nested EBV polymerase chain reaction to a LightCycler-based polymerase chain reaction resulted in a good correlation between both methods as demonstrated for standard plasmid DNA, reference DNA isolated from the EBV-genome containing Namalwa cell line, and DNA extracted from plasma/cerebrospinal fluid. However, a decrease of sensitivity by factor 10-100 was found in the LightCycler assay for DNA derived from peripheral blood mononuclear cells when larger amounts of background DNA were used presuming an inhibitory effect of cellular DNA. This was not observed in the nested EBV polymerase chain reaction. In order to be able to detect low copy numbers (10-100 EBV-genome copies/10^5 peripheral blood mononuclear cells) for better watching increasing EBV-genome copies screening of viral load in peripheral blood mononuclear cells is done in our laboratory by nested EBV polymerase chain reaction.

Material: Concerning the sample matrix testing of plasma, whole blood, leukocyte or mononuclear cell fractions are possible. Peripheral blood mononuclear cells are a useful material for measurement EBV load in transplant patients [Hoshino et al., 2001, Orentas et al., 2003]. However, whole blood combines all blood compartments that may harbor EBV [Stevens et al., 2001b]. Hakim et al. [2007] have found comparable sensitivities and a close quantitative correlation between EBV load in whole blood and peripheral blood mononuclear cells suggesting that a normalization to cell number in cellular specimens may not be necessary. Plasma as material for EBV polymerase chain reaction is favored by several authors [Orii et al., 2000, Niesters et al., 2000, Wagner et al., 2001]. In comparison to mononuclear cells the specificity for the diagnosis of EBV lymphoproliferative disease seems to be higher if plasma is taken for analysis as underlined by Wagner et al. [2001]. In contrast, the study performed by Hakim et al. [2007] demonstrated that whole blood and peripheral blood mononuclear cells are more sensitive than plasma alone when assayed for EBV load. In our experience, an increase in EBV-genome copies occurs earlier in peripheral blood mononuclear cells than in plasma and not all patients with EBV reactivation had positive plasma polymerase chain reaction results. Plasma polymerase chain reaction turned to be positive days to weeks later in patients with post-transplant lymphoproliferative disease, while copy numbers in peripheral blood mononuclear cells much earlier increased to >1,000 copies/10^5 peripheral blood mononuclear cells suggesting EBV reactivation. But plasma polymerase chain reaction might be of importance in the surveillance of therapy. Van Esser et al. [2001b] used the quantitative monitoring of EBV DNA levels in plasma for the prediction of the response to therapy of EBV post-transplant lymphoproliferative

disease after stem cell transplantation. Orii et al. [2000] concluded that antiviral therapy should be continued until qualitative plasma DNA is shown to be negative.

Cut-off: In the literature, the cut-off levels for the risk of post-transplant lymphoproliferative disease development vary considerably, e.g. between 200 copies/10^5 peripheral blood mononuclear cells [Green et al., 1999], ≥300/10^5 peripheral blood mononuclear cells [Cesaro et al., 2004], >300/µg DNA [Sirvent-von Bueltzingsloewen et al., 2002], >$10^{2,5}$/µg DNA [Hoshino et al., 2001], >500/75,000 peripheral blood mononuclear cells [Frias et al., 2001], >1,000/10^5 cells [Bacigalupo, 2005], >4,000/µg DNA [Wagner et al., 2004], 6,215/ml blood [Yancoski et al., 2004] and 10,000/µg DNA [Orentas et al., 2003]. As shown in Table 2, EBV load of >100,000 EBV-genome copies /10^5 peripheral blood mononuclear cells significantly predicts EBV lymphoproliferative disease in our study, whereas patients with ≤1,000 EBV-genome copies/10^5 peripheral blood mononuclear cells did not develop a lymphoproliferative disorder. The cut-off value of >1,000 EBV-genome copies in 10^5 peripheral blood mononuclear cells for the interpretation of EBV reactivation bases on our own experimental data with a lot of samples and was empirically stated. As depicted in Table 3, if an amount of viral load of >1,000 EBV-genome copies/10^5 peripheral blood mononuclear cells was taken for diagnosis EBV reactivation, sensitivity and specificity of this value was 100%, predictive values of positive and negative results was 1.00. If plasma was analyzed and a viral load of ≥500 EBV-genome copies/ml was used for the interpretation of active EBV infection, specificity reached 100% and predictive values for positive and negative results were 1,00. However, sensitivity yielded only 48.4% suggesting that screening of EBV load only in plasma is not sensitive enough for early intervention to prevent lymphoproliferative disease.

In the search for additional markers for predicting post-transplant lymphoproliferative disease, the determination of anti-EBV-specific T cell response as well as B lymphocytes should be taken into account. The results of several studies suggest that, in the future, the virus load measurement might be assisted by monitoring the EBV-specific immune response [Meij et al., 2003, Clave et al., 2004, Annels et al., 2006]. The authors measured the anti-EBV-specific T cell response in correlation to EBV load and the occurrence of EBV reactivation and lymphoproliferation, respectively in patients after allogeneic stem cell transplantation. Failure to detect EBV-specific $CD8^+$ T cells in patients with high-level reactivation was associated with the subsequent development of EBV post-transplant lymphoproliferative disease [Meij et al., 2003]. Controlling of EBV reactivation was characterized by rapid EBV-specific T lymphocyte increase concomitant to decreasing viral load [Clave et al., 2004]. Comparable to this observation two patients with post-transplant lymphoproliferative disease in the presented study showed after successful therapy a remarkable decrease of viral load accompanied with a complete recovery of clinical signs and an increase of total T cells, cytotoxic and helper T lymphocytes. The determination of specific circulating B lymphocyte population by flow cytometry is advocated as an additional predictive marker for development of post-transplant lymphoproliferative disease. The significant increase in B cell counts in patients with post-transplant lymphoproliferative disease presumably results from EBV-induced B cell proliferation [Levasseur et al., 2003]. Likewise, in patients 8 and 21 as well as in patient 22 with a lymphoproliferative disease of the presented study a B cell peak could be measured. Serial $CD19^+$ B lymphocyte measurements may also serve as a marker for clinical response during the treatment of post-transplant lymphoproliferative disease with the anti-B cell monoclonal antibody rituximab. Several studies have shown, that the treatment of post-transplant lymphoproliferative disease with rituximab resulted in reso-

lution of lymphoma. Clinical remission was accompanied by B cell depletion in the peripheral blood [McGuirk et al., 1999; Kuehnle et al., 2000; Ansell et al., 2002; Verschuuren et al., 2002; Levasseur et al., 2003] . Our own data confirm these observations. Two out of three patients with lymphoproliferative disease could be successfully treated with rituximab as part of the therapy occurring along with a decrease of B lymphocytes.

Regarding the therapeutic measures for prevention of EBV lymphoproliferative disease, modulation of immunosuppression based on EBV load is the first-line treatment. This resulted in a normalization of EBV levels in 24 out of 31 patients in the presented study. However, rapidly increasing EBV-genome copies in peripheral blood mononuclear cells together with EBV viremia call for pre-emptive therapy. It should consist of a combined treatment including reduction of immunosuppression, administration of antiviral drugs and immunotherapeutic agents, like cidofovir plus rituximab. Pre-emptive therapy with a single dose of rituximab led to the prevention of post-transplant lymphoproliferative disease and was first done by van Esser et al. [2002] and confirmed by Gruhn et al. [2003] and Dominietto et al. [2004]. The administration of rituximab induced a dramatic decrease of EBV-genome copies in peripheral blood mononuclear cells and plasma and effective depletion of B lymphocytes [Gruhn et al., 2003]. Rituximab was given to four patients of the presented study in combination with the antiviral agent cidofovir. Antivirals may have positive effects by inhibiting the lytic replication due to the suppression of proteins of the lytic cycle supporting the maintenance of lymphoproliferation [Montone et al. 1996]. In addition, detection of EBV DNA in plasma may indicate EBV viremia with the release of infectious virions. Inhibition of lytic virus production could inhibit the new-infection of B lymphocytes.

As underlined by Bacigalupo [2005] and confirmed in our own study, EBV reactivation is a very frequent event especially in patients undergoing alternative donor hematopoietic stem cell transplantation with antithymocyte globulin in the conditioning regimen. For this reason, patients should be monitored weekly by quantitative polymerase chain reaction because viral load is a significant predictor of post-transplant lymphoproliferative disease. Prevention of EBV lymphoproliferative disease by pre-emptive therapy based on molecular monitoring of EBV load is an attractive option to decrease the occurrence of post-transplant lymphoproliferative disease.

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Table 1: Clinical and laboratory data of patients with EBV reactivation after hematopoietic stem cell transplantation

Patient	Age (years)	Sex	Diagnosis	Conditioning regimen	Transplant donor	Transplant stem cell source	EBV reactivation day	EBV reactivation highest copy number per 10^5 PBMCs / ml plasma
Patients with post-transplant lymphoproliferative disease								
3	11	female	CML	FTBI, CY, ATG	MUD	S	+ 81	1.3 x 10^7 / 510,000
8	12	male	ALL	FTBI, VP, TH, ATG	MUD	S	+ 63	300,000 / 250,000
21	16	male	SLE	CY, FLU, PRED, ATG	A	S: $CD34^+$	+ 45	1.7 x 10^6 / 2,000
Patients receiving pre-emptive therapy								
20	14	female	MDS	BU, CY, MEL, ATG	MRD	S: $CD34^+$	+ 38	80,000 / 250,000
22	1	male	MDS	BU, CY, MEL, ATG	MUD	S: $CD34^+$	+ 39	600,000 / 1,000
23	9	female	JCA	CY, FLU, PRED, ATG	A	S: $CD34^+$	+ 36	80,000 / 2,000
95	19	male	GCT	(CIS, VP, IFO)□	A□	(S, B)□	-	-
				(BU, CY, FLU, TH, ATG)□□	MUD□□	S□□	+ 33□□	80,000 / 1,000
Patients with subclinical EBV reactivation								
43	12	female	MDS	FLU, TH, ATG	MUD	B	+ 49	10,000
45	3	female	ALL	FTBI, VP, ATG	MMUD	B	+ 97	5,000
52	12	female	CML	FTBI, CY, ATG	MUD	S: $CD34^+$	+ 75	10,000
53	20	female	SLE	CY, FLU, PRED, ATG	A	S: $CD34^+$	+ 25	10,000
64	1	male	NBL	CBPT, VP, MEL	A	S: $CD34^+$	+ 303	2,500
75	5	male	JMML	(BU, CY, MEL, ATG)✧	MUD✧	B✧	-	-
				ARA C✧✧	MUD✧✧	S✧✧	+ 49✧✧	2,500
76	17	female	ALL	FTBI, VP, ATG	MUD	B	+ 147	5,000
81	15	male	SAA	CY, ATG	MRD	B	+ 41	2,500
82	12	male	ALL	FTBI, CY, ATG	MUD	S	+ 46	2,500
84	17	male	AML	FTBI, CY, MEL, ATG	MUD	S	+ 104	2,500
88	20	male	ALL	FTBI, VP, ATG	MUD	B	+ 51	10,000

Table 1: ***continuation***

Patient	Age (years)	Sex	Diagnosis	Conditioning regimen	Transplant donor	Transplant stem cell source	EBV reactivation day	EBV reactivation highest copy number per 10^5 PBMCs / ml plasma
89	6	female	ALL	FTBI, VP	MRD	C	+ 252	2,500
97	8	male	AML	BU, CY, MEL	MRD	B	+ 63	2,500 / 1,000
98	22	female	ALL	FTBI, VP, ATG	MUD	B	+ 41	10,000 / 2,000
101	3	male	NBL	CBPT, VP, MEL	A	S: $CD34^+$	+ 19	40,000
102	13	female	CML	FTBI, CY, ATG	MMUD	S	+ 42	20,000 / 1,000
106	2	male	AML	BU, CY, MEL, ATG	MMUD	B	+ 87	40,000
107	6	male	NHL	FTBI, VP, TH, ATG	MMUD	B	+ 55	5,000 / 500
108	4	male	BT	(CBPT, VP, MTX)‡	$A^{\ddagger}$	$S^{\ddagger}$	-	-
				(CY, TH)‡‡	$A^{\ddagger\ddagger}$	$S^{\ddagger\ddagger}$	+ 21‡‡	40,000
112	6	male	ALL	FTBI, VP, ATG	MUD	B	+ 27	40,000 / 8,000
115	17	male	MDS	BU, CY, MEL, ATG	MUD	B	+ 58	10,000 / 1,000
117	8	female	ALL	FTBI, VP, ATG	MUD	B	+ 62	2,500 / 1,000
118	13	female	ALL	FTBI, VP, ATG	MUD	B	+ 55	20,000 / 500
120	12	male	ALL	FTBI, VP, ATG	MUD	B	+ 152	2,500

EBV: Epstein-Barr virus PBMCs: peripheral blood mononuclear cells

ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, BT: brain tumor, CML: chronic myeloid leukemia, JCA: juvenile chronic arthritis, JMML: juvenile myelomonocytic leukemia, GCT: germ cell tumor, MDS: myelodysplastic syndrome, NBL: neuroblastoma, NHL: non-Hodgkin lymphoma, SAA: severe aplastic anemia, SLE: systemic lupus erythematodes

ARA C: cytosine arabinoside, ATG: antithymocyte globulin, BU: busulphan, CBPT: carboplatine, CIS: cisplatine CY: cyclophosphamide, FLU: fludarabine, FTBI: fractionated total body irradiation, IFO: iphosphamide, MEL: melphalane, MTX: methotrexate, PRED: methylprednisolone, TH: thiotepa, VP: etoposide,

A: autologous, MRD: matched related donor, MUD: matched unrelated donor, MMUD: mismatched unrelated donor

B: bone marrow, S: stem cells, C: cord blood HSCT: hematopoietic stem cell transplantation

‡1. HSCT, ‡‡2. HSCT, $^{\square}$1. – 4. HSCT, $^{\square\square}$5. HSCT -: no EBV reactivation

Table 2: Comparison of results of EBV polymerase chain reaction with EBV-associated post-transplant lymphoproliferative disease

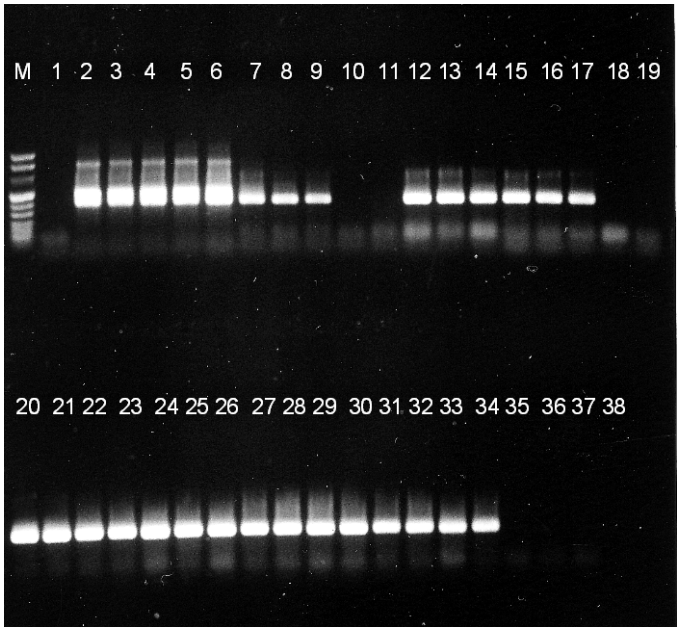
EBV-genome copies/10^5 PBMCs	EBV PCR in plasma	PTLD present (n=3)	PTLD absent (n=92)	Chi-square test	sensitivity (%)	specificity (%)	predictive value positive	predictive value negative
>100,000	positive	3	0	*P*<0.001	100	100	1.00	1.00
≤1,000	negative	0	92					

EBV: Epstein-Barr virus PBMCs: peripheral blood mononuclear cells PCR: polymerase chain reaction
PTLD: post-transplant lymphoproliferative disease

Table 3: Performance of EBV polymerase chain reaction for diagnosing EBV reactivation: comparison of peripheral blood mononuclear cells versus plasma as sample matrix

Patients	**EBV load >1,000 EBV-genome copies/10^5 PBMCs** (n)	**EBV load ≤1,000 EBV-genome copies/10^5 PBMCs** (n)	**EBV load ≥500 EBV-genome copies/ml plasma** (n)	**EBV load <500 EBV-genome copies/ml plasma** (n)
Patients with PTLD	3/3	0/3	3/3	0/3
Patients at very high risk for PTLD → pre-emptive therapy	4/4	0/4	4/4	0/4
Patients at risk for PTLD → immunomodulation	24/24	0/24	8/24	16/24
Patients without signs of active EBV infection	0/92	92/92	0/92	92/92

EBV: Epstein-Barr virus PBMCs: peripheral blood mononuclear cells PCR: polymerase chain reaction
PTLD: post-transplant lymphoproliferative disease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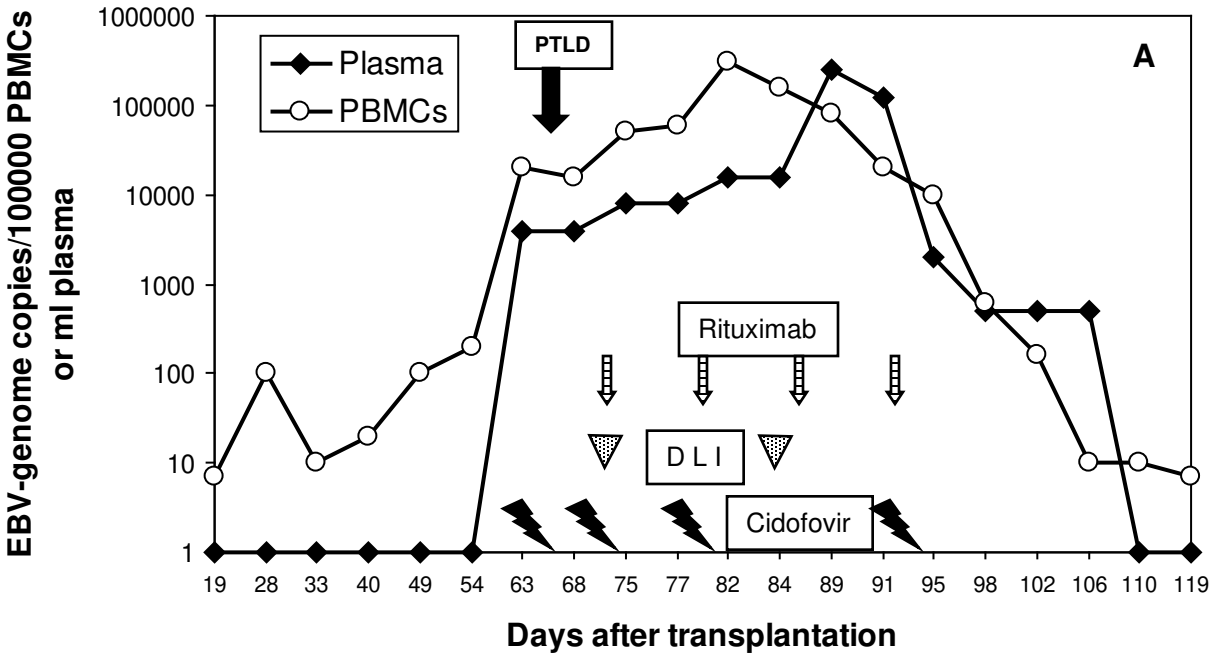
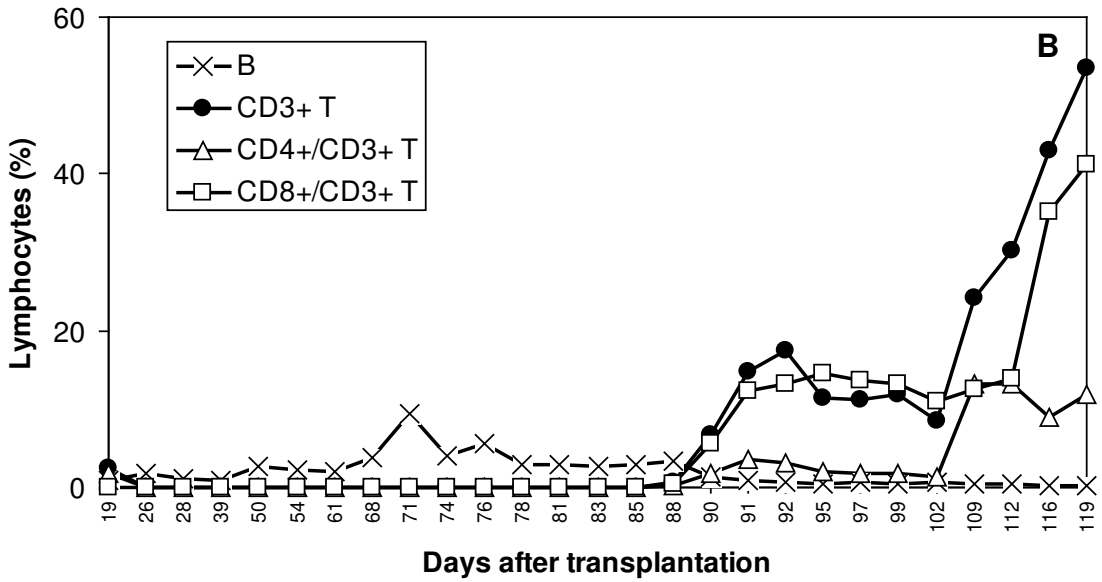
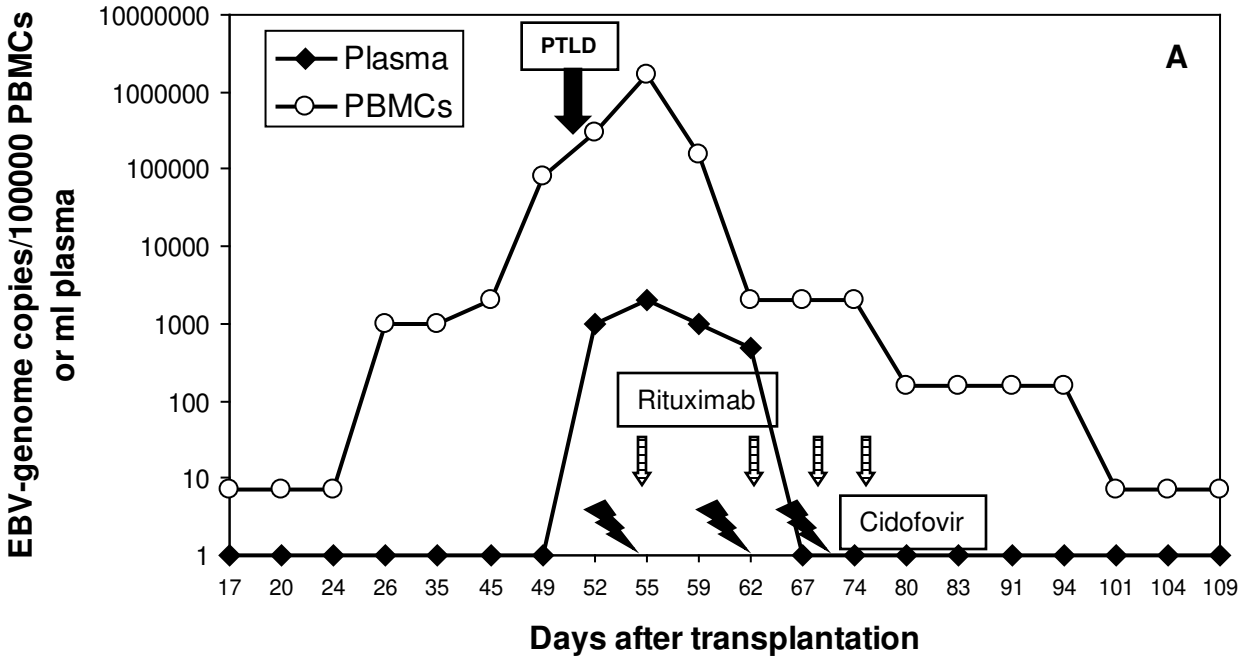
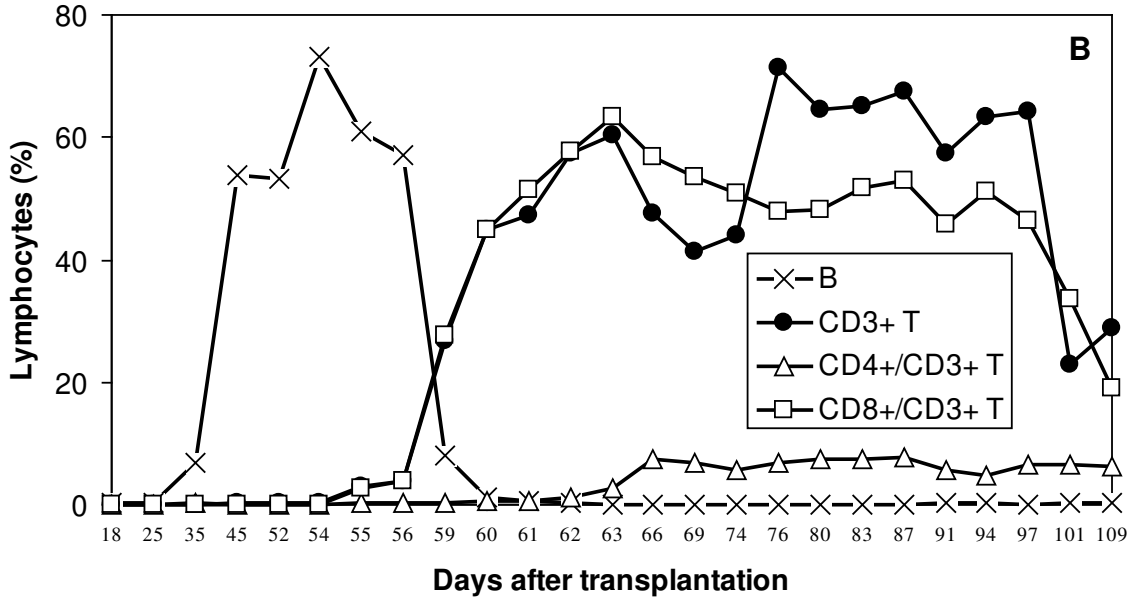
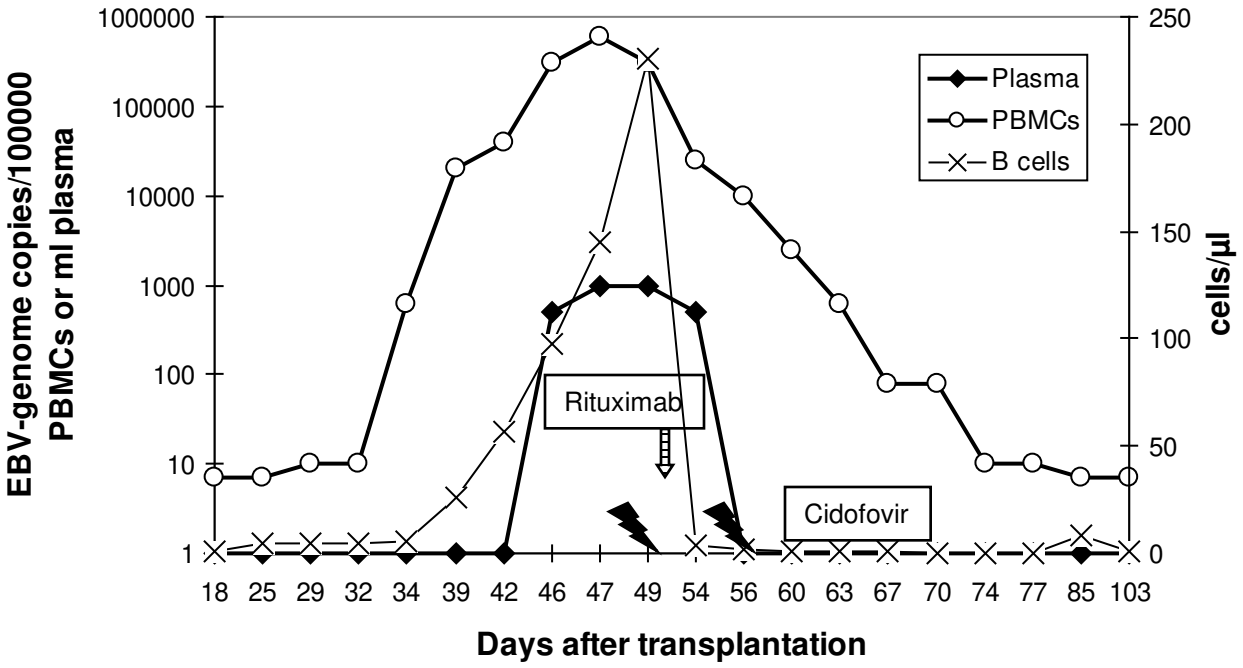
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38


EBV-genome copies/100000 PBMCs or ml plasma
1000000
100000
10000
1000
100
10
1
Plasma
PBMCs
PTLD
A
Rituximab
D L I
Cidofovir
19 28 33 40 49 54 63 68 75 77 82 84 89 91 95 98 102 106 110 119
Days after transplantation


Lymphocytes (%)
60
40
20
0
B
CD3+ T
CD4+/CD3+ T
CD8+/CD3+ T
B
19 26 28 39 50 54 61 68 71 74 76 78 81 83 85 88 90 91 92 95 97 99 102 109 112 116 119
Days after transplantation


EBV-genome copies/100000 PBMCs or ml plasma
10000000
1000000
100000
10000
1000
100
10
1
Plasma
PBMCs
PTLD
A
Rituximab
Cidofovir
17 20 24 26 35 45 49 52 55 59 62 67 74 80 83 91 94 101 104 109
Days after transplantation


Lymphocytes (%)
80
60
40
20
0
B
B
CD3+ T
CD4+/CD3+ T
CD8+/CD3+ T
18 25 35 45 52 54 55 56 59 60 61 62 63 66 69 74 76 80 83 87 91 94 97 101 109
Days after transplantation


EBV-genome copies/100000 PBMCs or ml plasma
1000000
100000
10000
1000
100
10
1
250
200
150
100
50
0
cells/µl
Plasma
PBMCs
B cells
Rituximab
Cidofovir
18 25 29 32 34 39 42 46 47 49 54 56 60 63 67 70 74 77 85 103
Days after transplantation

Legends to the figures

Figure 1:

Agarose gel electrophoresis of Epstein-Barr virus (EBV) nested polymerase chain reaction (PCR) of plasma and peripheral blood mononuclear cells (PBMCs) of patient 8, day +84. **Lane M:** DNA molecular weight marker (VIII, Roche), Lane 1: DNA from Ramos cells (100 ng), **Lane 2-11:** EBV plasmid (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0, 10^{-1} copies/PCR), **Lane 12-19:** DNA from plasma (undiluted, corresponding to a DNA equivalent of 20 µl plasma, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128), **Lane 20-37:** DNA from PBMCs (1 µg, 500 ng, 250 ng, 125 ng, 62 ng, 31 ng, 15 ng, 8 ng, 4 ng, 2 ng, 1 ng, 500 pg, 250 pg, 125 pg, 62 pg, 31 pg 15 pg, 8 pg), **Lane 38:** Control without DNA. Using nested EBV-specific PCR the EBV genome can still be detected after amplification of 10 EBV plasmid copies (lane 9). In patient 8, who developed an EBV-associated post-transplant lymphoproliferative disease, 16,000 EBV-genome copies/ml plasma and 160,000 EBV-genome copies/100,000 PBMCs could be detected.

Figure 2A, B:

Epstein-Barr virus (EBV) load in plasma and peripheral blood mononuclear cells (PBMCs). Therapeutic intervention during the course of EBV-associated post-transplant lymphoproliferative disease (PTLD) in patient 8 (2A). Percentage of lymphocytes during the course of illness (2B). The high EBV load in plasma and PBMCs reduced dramatically after therapy. The decrease of EBV-genome copies was associated with a decrease of B lymphocytes and an increase of $CD3^+$, $CD8^+/CD3^+$ and $CD4^+/CD3^+$ T lymphocytes.

D L I: donor lymphocyte infusion.

Figure 3A, B:

Epstein-Barr virus (EBV)-genome copies in plasma and peripheral blood mono-nuclear cells (PBMCs). Therapeutic intervention during the course of EBV-associated post-transplant lymphoproliferative disease (PTLD) in patient 21 (3A). Percentage of lymphocytes during the course of illness (3B). B cell proliferation was in line with the increasing EBV load. After combined therapy with cidofovir and rituximab EBV load reduced remarkably. The decline of EBV-genome copies and B cells was accompanied by a reconstitution of T-cell immunity.

Figure 4:

Epstein-Barr-virus (EBV)-genome copies and number of B lymphocytes in patient 22 before and after pre-emptive therapy with rituximab and cidofovir. After pre-emptive therapy a dramatic decrease of EBV load as well as B lymphocytes occurred.